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Invention: L-LYSINE-PRODUCING CORYNEBACTERIA AND PROCESS FOR THE PREPARATION OF LYSINE

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SPECIFICATION

L-Lysine-Producing Corynebacteria and Process for the Preparation of Lysine

This application is a continuation-in-part of U.S. application no. 09/353,608, filed July 14,1999, now abandoned which is incorporated herein by reference in its entirely. DJS 6/3/03

BACKGROUND OF THE INVENTION

Field of the Invention

The invention relates to L-lysine-producing strains of corynebacteria with enhanced pyc gene (pyruvate carboxylase gene), in which strains additional genes, chosen from the group consisting of the dapA gene (dihydrodipicolinate synthase gene), the lysC gene (aspartate kinase gene), the lysE gene (lysine export carrier gene) and the dapB gene (dihydrodipicolinate reductase gene), but especially the dapA gene, are enhanced and, in particular, over-expressed, and to a process for the preparation of L-lysine.

Background Information

L-Lysine is a commercially important L-amino acid which is used especially as a feed additive in animal nutrition. The demand of such feed additives has been steadily increasing in recent years.

L-Lysine is prepared by a fermentation process with L-lysine-producing strains of corynebacteria, especially Corynebacterium glutamicum. Because of the great importance of this product, attempts are constantly being made to improve the preparative process. Improvements to the process may relate to measures involving the fermentation technology, e.g. stirring and oxygen supply, or the composition of the nutrient media, e.g. the sugar concentration during fermentation, or the work-up to the product form, e.g. by ion exchange chromatography, or the

intrinsic productivity characteristics of the microorganism itself.

The productivity characteristics of these microorganisms are improved by using methods of mutagenesis, selection and mutant choice to give strains which are resistant to antimetabolites, e.g. S-(2-aminoethyl)cysteine, or auxotrophic for amino acids, e.g. L-leucine, and produce L-lysine.

Methods of recombinant DNA technology have also been used for some years in order to improve L-lysine-producing strains of Corynebacterium glutamicum by amplifying individual biosynthesis genes and studying the effect on the L-lysine production.

Thus, EP-A-0 088 166 reports the increase in productivity, after amplification, of a DNA fragment conferring resistance to aminoethylcysteine. EP-B-0 387 527 reports the increase in productivity, after amplification, of an lysC allele coding for a feedback-resistant aspartate kinase. EP-B-0 197 335 reports the increase in productivity, after amplification, of the dapA gene coding for dihydrodipicolinate synthase. EP-A-0 219 027 reports the increase in productivity, after amplification, of the asd gene coding for aspartate semialdehyde dehydrogenase. Pisabarro et al. (Journal of Bacteriology 175(9), 2743-2749 (1993)) describe the dapB gene coding for dihydrodipicolinate reductase.

The effect of the amplification of primary metabolism genes on the L-lysine production has also been studied. Thus EP-A-0 219 027 reports the increase in productivity, after amplification, of the aspC gene coding for aspartate aminotransferase. EP-B-0 143 195 and EP-B-0 358 940 report the increase in productivity, after amplification, of the ppc gene coding for phosphoenolpyruvate carboxylase. DE-A-198 31 609 reports the increase in

productivity, after amplification, of the pyc gene coding for pyruvate carboxylase. The anaplerotic reaction catalyzed by pyruvate carboxylase is of particular importance compared with the reaction catalyzed by phosphoenolpyruvate carboxylase. Thus, Wendisch et al. (FEMS Microbiology Letters 112, 269-274 (1993)) showed that the lysine production of the strain MH20-22B was not impaired by turning off the ppc gene.

Finally, DE-A-195 48 222 describes that an increased activity of the L-lysine export carrier coded for by the lysE gene promotes lysine production.

In addition to these attempts to amplify an individual gene, attempts have also been made to amplify two or more genes simultaneously, thereby improving the L-lysine production in corynebacteria. Thus, DE-A-38 23 451 reports the increase in productivity, after simultaneous amplification, of the asd gene and the dapA gene from Escherichia coli. DE-A-39 43 117 discloses the increase in productivity, after simultaneous amplification, of an lysC allele coding for a feedback-resistant aspartate kinase and of the dapA gene. EP-A-0 841 395 particularly reports the increase in productivity, after simultaneous amplification, of an lysC allele coding for a feedback-resistant aspartate kinase and of the dapB gene; further improvements could be achieved by additional amplification of the dapB, lysA and ddh genes. EP-A-0 854 189 describes the increase in productivity, after simultaneous amplification, of an lysC allele coding for a feedback-resistant aspartate kinase and of the dapA, dapB, lysA and aspC genes. EP-A-0 857 784 particularly reports the increase in productivity, after simultaneous amplification, of an lysC allele coding for a feedback-resistant aspartate kinase enzyme and the lysA gene; a further improvement could be achieved by additional amplification of the ppc gene.

It is clear from the many processes described in the state of the art, that there is a need for the development of novel approaches and for the improvement of existing processes for lysine production with corynebacteria.

SUMMARY OF THE INVENTION

Object of the Invention

It is an object of the invention to provide novel L-lysine-producing strains of corynebacteria and processes for the preparation of L-lysine.

Description of the Invention

When L-lysine or lysine is mentioned in the following text, it should be understood as meaning not only the base but also the appropriate salts, e.g. lysine hydrochloride or lysine sulfate.

The invention provides L-lysine-producing strains of corynebacteria with enhanced pyc gene (pyruvate carboxylase gene), wherein additional genes, chosen from the group consisting of the dapA gene (dihydrodipicolinate synthase gene), the lysC gene (aspartate kinase gene), the lysE gene (lysine export carrier gene) and the dapB gene (dihydrodipicolinate reductase gene), but especially the dapA gene, are enhanced and, in particular, over-expressed.

A novel DNA sequence located upstream (5' end) from the dapB gene has also been found, which carries the -35 region of the dapB promoter and is advantageous for the expression of the dapB gene. It is shown as SEQ ID No. 1.

A corresponding DNA capable of replication, with the nucleotide sequence shown in SEQ ID No. 1, is also included in the invention.

The invention also provides the MC20 and MA16 mutations of the dapA promoter shown in SEQ ID No. 5 and SEQ ID No. 6, deposited in the strains DSM12868 and DSM12867.

The invention furthermore provides L-lysine-producing strains of corynebacteria with amplified pyc gene, wherein

additionally the dapA and dapB genes are simultaneously enhanced and, in particular, over-expressed.

The invention also provides L-lysine-producing strains of corynebacteria with enhanced pyc gene, wherein additionally the dapA, dapB and lysE genes are simultaneously enhanced and, in particular, over-expressed.

In this context the term "enhancement" describes the increase in the intracellular activity, in a microorganism, of one or more enzymes which are coded for by the appropriate DNA, by increasing the copy number of the gene(s), using a strong promoter or using a gene coding for an appropriate enzyme with a high activity, and optionally combining these measures.

In this context, "amplification" describes a specific procedure for achieving an enhancement whereby the number of DNA molecules carrying a gene or genes, an allele or alleles, a regulatory signal or signals or any other genetic feature(s) is increased.

The invention also provides a process for the preparation of L-lysine using the bacteria described above.

The microorganisms which the present invention provides can prepare L-lysine from glucose, sucrose, lactose, fructose, maltose, molasses, starch or cellulose or from glycerol and ethanol, especially from glucose or sucrose. Said microorganisms are corynebacteria, especially of the genus Corynebacterium. The species Corynebacterium glutamicum may be mentioned in particular in the genus Corynebacterium, being known to those skilled in the art for its ability to produce amino acids. This species includes wild-type strains such as Corynebacterium glutamicum ATCC13032, Brevibacterium flavum ATCC14067, Corynebacterium melassecola ATCC17965 and strains or

mutants derived therefrom. Examples of L-lysine-producing mutants of corynebacteria are:

Corynebacterium glutamicum FERM-P 1709
Brevibacterium flavum FERM-P 1708
Brevibacterium lactofermentum FERM-P 1712
Brevibacterium flavum FERM-P 6463
Brevibacterium flavum FERM-P 6464
Corynebacterium glutamicum DSM5714
Corynebacterium glutamicum DSM12866

It has now been found that an enhanced expression of the lysE gene in addition to the pyc gene, or an additionally enhanced expression of an lysC allele coding for a feedback-resistant aspartate kinase, or an additionally enhanced expression of the dapB gene and, in particular, an additionally enhanced expression of the dapA gene, individually or together, further improve L-lysine production.

It has also been found that, for a given over-expression of the pyc gene, the simultaneous, additionally enhanced expression of the dapA and dapB genes brings further advantages for L-lysine production.

Finally, it has been found that, for a given over-expression of the pyc gene, the simultaneous, additionally enhanced expression of the dapA, dapB and lysE genes is extremely advantageous for L-lysine production.

An enhancement (over-expression) is achieved, e.g., by increasing the copy number of the appropriate genes or mutating the promoter and regulatory region or the ribosome binding site located upstream from the structural gene. Expression cassettes incorporated upstream from the structural gene function in the same way. Inducible promoters additionally make it possible to increase the expression in the course of the formation of L-lysine by fermentation. Measures for prolonging the life of the

mRNA also improve the expression. Furthermore, the enzyme activity is also enhanced by preventing the degradation of the enzyme protein, the genes or gene constructs either being located in plasmids (shuttle vectors) of variable copy number or being integrated and amplified in the chromosome. Alternatively, it is also possible to achieve an over-expression of the genes in question by changing the composition of the media and the culture technique.

Those skilled in the art will find relevant instructions inter alia in Martin et al. (Bio/Technology 5, 137-146 (1987)), Guerrero et al. (Gene 138, 35-41 (1994)), Tsuchiya and Morinaga (Bio/Technology 6, 428-430 (1988)), Eikmanns et al. (Gene 102, 93-98 (1991)), EP-0 472 869, US 4,601,893, Schwarzer and Pühler (Bio/Technology 9, 84-87 (1991)), Reinscheid et al. (Applied and Environmental Microbiology 60, 126-132 (1994)), LaBarre et al. (Journal of Bacteriology 175, 1001-1007 (1993)), WO 96/15246, Malumbres et al. (Gene 134, 15-24 (1993)), JP-A-10-229891, Jensen and Hammer (Biotechnology and Bioengineering 58, 191-195 (1998)) or the handbook "Manual of Methods for General Bacteriology" of the American Society for Bacteriology (Washington DC, USA, 1981) and well-known textbooks on genetics and molecular biology.

The genes from Corynebacterium glutamicum used according to the invention are described and can be isolated, prepared or synthesized by known methods.

Methods of localized mutagenesis are described, *inter alia*, by Higuchi et al. (Nucleic Acids Research 16, 7351-7367 (1988)) or by Silver et al. in the handbook by Innis, Glefand and Sninsky (eds.) entitled "PCR Strategies" (Academic Press, London, UK, 1995).

The first step in isolating a gene of interest from C. glutamicum is to construct a gene library of this microorganism in, e.g., E. coli or optionally also in C.

glutamicum. The construction of gene libraries is documented in generally well-known textbooks and handbooks. Examples which may be mentioned are the textbook by Winnacker entitled "From Genes to Clones, Introduction to Gene Technology" (Verlag Chemie, Weinheim, Germany, 1990) or the handbook by Sambrook et al. entitled "Molecular Cloning, A Laboratory Manual" (Cold Spring Harbor Laboratory Press, 1989). Bathe et al. (Molecular and General Genetics, 252: 255-265 (1996)) describe a gene library of C. glutamicum ATCC13032 which was constructed using cosmid vector SuperCos I (Wahl et al., Proceedings of the National Academy of Sciences, USA 84, 2160-2164 (1987)) in E. coli K-12 NM554 (Raleigh et al., Nucleic Acids Research 16: 1563-1575 (1988)). Börmann et al. (Molecular Microbiology 6(3), 317-326) in turn describe a gene library of C. glutamicum ATCC13032 using cosmid pHC79 (Hohn and Collins, Gene 11, 291-298 (1980)). A gene library of C. glutamicum in E. coli can also be constructed using plasmids like pBR322 (Bolivar, Life Sciences 25, 807-818 (1979)) or pUC19 (Norrander et al., Gene 26: 101-106 (1983)). In the same way, it is also possible to use shuttle vectors such as pJC1 (Cremer et al., Molecular and General Genetics 220, 478-480 (1990)) or pEC5 (Eikmanns et al., Gene 102, 93-98 (1991)), which replicate in E. coli and C. glutamicum. Restriction- and/or recombination-defective strains are particularly suitable hosts, an example being the E. coli strain DH5αmcr, which has been described by Grant et al. (Proceedings of the National Academy of Sciences, USA 87, 4645-4649 (1990)). Other examples are the restriction-defective C. glutamicum strains RM3 and RM4, which are described by Schäfer et al. (Applied and Environmental Microbiology 60(2), 756-759 (1994)).

The gene library is then transferred to an indicator strain by transformation (Hanahan, Journal of Molecular Biology 166, 557-580 (1983)) or electroporation (Tauch et

al., FEMS Microbiological Letters, 123: 343-347 (1994)). The characteristic feature of the indicator strain is that it possesses a mutation in the gene of interest which causes a detectable phenotype, e.g. an auxotrophy. The indicator strains or mutants are obtainable from publicized sources or strain collections, e.g. the Genetic Stock Center of Yale University (New Haven, Connecticut, USA), or if necessary are specially prepared. An example of such an indicator strain which may be mentioned is the E. coli strain RDA8 requiring mesodiaminopimelic acid (Richaud et al., C.R. Acad. Sci. Paris Ser. III 293: 507-512 (1981)), which carries a mutation (dapA::Mu) in the dapA gene.

After transformation of the indicator strain with a recombinant plasmid carrying the gene of interest, and expression of the gene in question, the indicator strain becomes prototrophic in respect of the appropriate characteristic. If the cloned DNA fragment confers resistance, e.g. to an antimetabolite such as S-(2-aminoethyl)cysteine, the indicator strain carrying the recombinant plasmid can be identified by selection on appropriately supplemented nutrient media.

If the nucleotide sequence of the gene region of interest is known or obtainable from a data bank, the chromosomal DNA can be isolated by known methods, e.g. as described by Eikmanns et al. (Microbiology 140, 1817-1828 (1994)), and the gene in question can be synthesized by the polymerase chain reaction (PCR) using suitable primers and cloned into a suitable plasmid vector, e.g. pCRIITOPO from Invitrogen (Groningen, The Netherlands). A summary of PCR methodology can be found in the book by Newton and Graham entitled "PCR" (Spektrum Akademischer Verlag, Heidelberg, Germany, 1994).

Examples of publicly accessible data banks for nucleotide sequences are that of the European Molecular Biologies

Laboratories (EMBL, Heidelberg, Germany) or that of the National Center for Biotechnology Information (NCBI, Bethesda, MD, USA).

The isolation and cloning of the pyc gene from C. glutamicum ATCC13032 are described in DE-A-198 31 609 and by Koffas et al. (Applied Microbiology and Biotechnology 50, 346-352 (1988)). The nucleotide sequence of the pyc gene is obtainable under accession number AF038548 or Y09548.

The isolation and cloning of the lysE gene from C. glutamicum ATCC13032 are described in DE-A-195 48 222. The nucleotide sequence of the lysE gene is obtainable under accession number X96471.

The isolation, cloning and sequencing of the dapA gene from various strains of C. glutamicum are described by Cremer et al. (Molecular and General Genetics 220: 478-480 (1990)), by Pisabarro et al. (Journal of Bacteriology 175: 2743-2749 (1993)) and by Bonnassie et al. (Nucleic Acids Research 18: 6421 (1990)). DE-A-39 43 117 reports the amplification of the dapA gene by means of plasmid pJC23. The nucleotide sequence of the dapA gene is obtainable under accession number X53993.

The isolation, cloning and sequencing of the dapB gene from Brevibacterium lactofermentum are described by Pisabarro et al. (Journal of Bacteriology 175: 2743-2749 (1993)). The nucleotide sequence of the dapB gene is obtainable under accession number X67737.

The isolation, cloning and sequencing of the lysC gene and of lysC alleles coding for a feedback-resistant aspartate kinase are reported by several authors. Thus, Kalinowski et al. (Molecular and General Genetics 224: 317-324 (1990)) report the lysC allele from the C. glutamicum strain DM58-1. DE-A-39 43 117 reports the cloning of the lysC allele from the C. glutamicum strain MH20. Follettie

et al. (Journal of Bacteriology 175: 4096-4103 (1993)) report the lysC allele from the C. flavum strain N13, which is called "ask" in said publication. The nucleotide sequences of the lysC gene and of various lysC alleles are obtainable inter alia under accession numbers X57226 and E06826.

The genes obtained in this way can then be incorporated inter alia into plasmid vectors, e.g. pJC1 (Cremer et al., Molecular and General Genetics 220, 478-480 (1990)) or pEC5 (Eikmanns et al., Gene, 102, 93-98 (1991)), individually or in suitable combinations, transferred to desired strains of corynebacteria, e.g. the strain MH20-22B (Schrumpf et al., Applied Microbiology and Biotechnology 37: 566-571 (1992)), by transformation, e.g. as in Thierbach et al. (Applied Microbiology and Biotechnology 29, 356-362 (1988)), or by electroporation, e.g. as in Dunican and Shivnan (Bio/Technology 7, 1067-1070 (1989)), and expressed. The strain to be chosen can equally well be transformed with two plasmid vectors, each containing the gene or genes in question, thereby achieving the advantageous, simultaneously enhanced expression of two or more genes in addition to the known enhancement of the pyc gene.

Examples of such strains are:

- the strain MH20-22B/pJC23/pEC7pyc, in which the pyc and dapA genes are simultaneously enhanced, or
- the strain MH20-22B/pJC33/pEC7pyc, in which the pyc- and the lysC(FBR) allele are simultaneously enhanced and, in particular, over-expressed, or
- the strain MH20-22B/pJC23/pEC7dapBpyc, in which the pyc, dapA and dapB genes are simultaneously enhanced and, in particular, over-expressed, or

- the strain MH20-22B/pJC23/pEC7lysEdapBpyc, in which the pyc, dapA, dapB and lysE genes are simultaneously enhanced and, in particular, over-expressed.

The microorganisms prepared according to the invention can be cultivated for L-lysine production continuously or discontinuously by the batch process, the fed batch process or the repeated fed batch process. A summary of known cultivation methods is provided in the textbook by Chmiel (Bioprozesstechnik 1. Einführung in die Bioverfahrenstechnik (Bioprocess Technology 1. Introduction to Bioengineering) (Gustav Fischer Verlag, Stuttgart, 1991)) or in the textbook by Storhas (Bioreaktoren und periphere Einrichtungen (Bioreactors and Peripheral Equipment) (Vieweg Verlag, Brunswick/Wiesbaden, 1994)).

The culture medium to be used must appropriately meet the demands of the particular microorganisms. Descriptions of culture media for various microorganisms can be found in the handbook "Manual of Methods for General Bacteriology" of the American Society for Bacteriology (Washington D.C., USA, 1981). Carbon sources which can be used are sugars and carbohydrates, e.g. glucose, sucrose, lactose, fructose, maltose, molasses, starch and cellulose, oils and fats, e.g. soya oil, sunflower oil, groundnut oil and coconut fat, fatty acids, e.g. palmitic acid, stearic acid and linoleic acid, alcohols, e.g. glycerol and ethanol, and organic acids, e.g. acetic acid. These substances can be used individually or as a mixture. Nitrogen sources which can be used are organic nitrogen-containing compounds such as peptones, yeast extract, meat extract, malt extract, corn steep liquor, soybean flour and urea, or inorganic compounds such as ammonium sulfate, ammonium chloride, ammonium phosphate, ammonium carbonate and ammonium nitrate. The nitrogen sources can be used individually or as a mixture. Phosphorus sources which can be used are potassium dihydrogenphosphate or

dipotassium hydrogenphosphate or the corresponding sodium salts. The culture medium must also contain metal salts, e.g. magnesium sulfate or iron sulfate, which are necessary for growth. Finally, essential growth-promoting substances such as amino acids and vitamins can be used in addition to the substances mentioned above. Said feed materials can be added to the culture all at once or fed in appropriately during cultivation.

The pH of the culture is controlled by the appropriate use of basic compounds such as sodium hydroxide, potassium hydroxide or ammonia, or acid compounds such as phosphoric acid or sulfuric acid. Foaming can be controlled using antifoams such as fatty acid polyglycol esters. The stability of plasmids can be maintained by adding suitable selectively acting substances, e.g. antibiotics, to the medium. Aerobic conditions are maintained by introducing oxygen or oxygen-containing gaseous mixtures, e.g. air, into the culture. The temperature of the culture is normally 20°C to 45°C and preferably 25°C to 40°C. The culture is continued until L-lysine formation has reached a maximum. This objective is normally achieved within 10 hours to 160 hours.

The concentration of L-lysine formed can be determined with the aid of amino acid analyzers by means of ion exchange chromatography and postcolumn reaction with ninhydrin detection, as described by Spackmann et al. (Analytical Chemistry 30, 1190 (1958)).

The following microorganisms have been deposited at the Deutsche Sammlung für Mikroorganismen und Zellkulturen (German Collection of Microorganisms and Cell Cultures (DSMZ), Brunswick, Germany) under the terms of the Budapest Treaty:

- Escherichia coli K-12 strain DH5α/pEC7pyc as DSM12870

- Escherichia coli K-12 strain DH5α/pEC7dapBpyc as DSM12873
- Escherichia coli K-12 strain DH5α/pEC7lysEdapBpyc as DSM12874
- Corynebacterium glutamicum strain DSM5715/pJC23 as DSM12869
- Corynebacterium glutamicum strain DSM5715aecD::dapA(MA16) as DSM12867
- Corynebacterium glutamicum strain DSM5715aecD::dapA(MC20) as DSM12868
- Corynebacterium glutamicum strain DM678 as DSM12866
- Escherichia coli K-12 strain DH5α/pEC7lysEpyc as DSM 12872
- Escherichia coli K-12 strain DH5α/pEC7dapBlysE as DSM 12875
- Escherichia coli K-12 strain DH5α/pEC7lysE as DSM 12871

DETAILED DESCRIPTION OF THE INVENTION

The present invention is illustrated in greater detail below with the aid of Examples.

Example 1

Preparation of the DNA coding for the lysE gene

Chromosomal DNA was isolated from the strain ATCC13032 by the conventional methods (Eikmanns et al., Microbiology 140: 1817-1828 (1994)). The polymerase chain reaction (PCR) was used to amplify a DNA fragment carrying the lysE gene. The following primer oligonucleotides were chosen for the PCR on the basis of the lysE gene sequence known

for C. glutamicum (Vrljic et al., Molecular Microbiology 22(5), 815-826 (1996)) (accession number X96471):

LysBam1:
5′ CTC GAG AGC (GGA TCC) GCG CTG ACT CAC C 3′

LysBam2:
5′ GGA GAG TAC GGC (GGA TCC) ACC GTG ACC 3′

The primers shown were synthesized by MWG Biotech (Ebersberg, Germany) and the PCR was carried out by the standard PCR method of Innis et al. (PCR Protocols. A Guide to Methods and Applications, 1990, Academic Press). The primers make it possible to amplify an approx. 1.1 kb DNA fragment carrying the lysE gene. The primers also contain the sequence for the cleavage site of the restriction endonuclease BamHI, which is indicated by brackets in the nucleotide sequence shown above.

The amplified DNA fragment of approx. 1.1 kb, carrying the lysE gene, was identified by means of electrophoresis in 0.8% agarose gel, isolated from the gel and purified with the QIAquick Gel Extraction Kit (cat. no. 28704) from Qiagen (Hilden, Germany).

The fragment was then ligated by means of T4 DNA ligase from Boehringer Mannheim (Mannheim, Germany) to vector pUC18 (Norrander et al., Gene (26) 101-106 (1983)). This was done by fully cleaving vector pUC18 with the restriction endonuclease SmaI and treating it with alkaline phosphatase (Boehringer Mannheim, Mannheim, Germany). The ligation mixture was transformed to the E. coli strain DH5α (Hanahan, in: DNA Cloning. A Practical Approach, Vol. I, IRL-Press, Oxford, Washington DC, USA). Plasmid-carrying cells were selected by plating the transformation mixture on LB agar (Sambrook et al., Molecular Cloning: A Laboratory Manual, 2nd ed., Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.) which had been supplemented with 50 mg/l of ampicillin.

Plasmid DNA was isolated from a transformant and checked by treatment with the restriction enzyme BamHI followed by agarose gel electrophoresis. The plasmid was called pUC18lysE.

Example 2

Preparation of the dapB gene

Chromosomal DNA was isolated from the Corynebacterium glutamicum strain ATCC13032 as indicated in Example 1. The sequence of the dapB gene as such from Corynebacterium glutamicum is known (accession number X67737). However, the published DNA sequence comprises only 56 bp upstream from the translation start, so the 5' end upstream from the translation start was additionally sequenced.

The sequencing was carried out with plasmid pJC25 (EP-B 0 435 132) using a primer oligonucleotide which binds in the region of the known dapB sequence (accession number X67737). The sequence of the sequencing primer used was:

~~5' GAA CGC CAA CCT TGA TTC C 3'~~

The sequencing was carried out by the chain termination method described by Sanger et al., Proc. Natl. Acad. Sci. USA, (74) 5463-5467 (1977). The sequencing reaction was performed with the aid of the AutoRead Sequencing Kit (Pharmacia, Freiburg). The electrophoretic analysis and detection of the sequencing products were carried out with the A.L.F. DNA sequencer from Pharmacia (Freiburg, Germany).

The DNA sequence obtained was used to choose a second primer in order to obtain further sequence data upstream from the transcription start. The following primer was chosen for this purpose:

~~5' CTT TGC CGC CGT TGG GTT C 3'~~

The sequencing reaction was carried out as described above. The novel sequence upstream from the dapB gene is shown as SEQ ID No. 1. The sequence including the nucleotide sequence of the dapB gene is shown as SEQ ID No. 2.

The polymerase chain reaction was used to amplify the dapB gene. For this purpose, two primer oligonucleotides, chosen on the basis of the known DNA sequence of the dapB gene, were synthesized by MWG Biotech:

P-dap:
5′ (AAG CTT) AGG TTG TAG GCG TTG AGC 3′

dapall:
5′ TTA ACT TGT TCG GCC ACA GC 3′

The 5′ primer (primer P-dap) contains a HindIII cleavage site which is indicated by brackets in the sequence shown above. The PCR was carried out as in Example 1. An approx. 1.1 kb DNA fragment, which carries the dapB gene and contains a cleavage site for the restriction endonuclease HindIII at each end, was amplified in this way. The PCR fragment obtained was purified from 0.8% agarose gel (QIAquick Gel Extraction Kit from Qiagen, Hilden, Germany) and cloned into cloning vector pCR2.1TOPO (Invitrogen, Leek, The Netherlands) with the TOPO TA Cloning Kit (Invitrogen, Leek, The Netherlands, cat. no. K4550-01). The ligation mixture was transformed to the E. coli strain TOP10F′ from Invitrogen, the transformation mixture was plated on LB agar containing kanamycin (50 mg/l), IPTG (0.16 mM) and X-Gal (64 mg/l) and kanamycin-resistant, white colonies were isolated. Plasmid DNA was isolated from a transformant with the aid of the QIAprep Spin Miniprep Kit from Qiagen and checked by cleavage with the restriction enzyme HindIII followed by agarose gel electrophoresis. The DNA sequence of the amplified DNA fragment was checked by sequencing. The sequence of the

PCR product matches the sequence shown in SEQ ID No. 1. The plasmid obtained was called pCR2.1TOPOdapB.

Example 3

Preparation of the DNA coding for the pyc gene

The Corynebacterium glutamicum strain ATCC13032 was used as the donor for the chromosomal DNA. Chromosomal DNA was isolated from the strain ATCC13032 as described in Example 1. The polymerase chain reaction was used to amplify a DNA fragment carrying the pyc gene. The following primer oligonucleotides were chosen for the PCR on the basis of the pyc gene sequence known for C. glutamicum (Peters-Wendisch et al., Microbiology 144, 915-927 (1998)) (accession number Y09548):

5-PYC-IN:
5' GC(T CTA GA)A GTG TCG CAA CCG TGC TTG A 3'

3-PYC-IN:
5' GC(T CTA GA)T TGA GCC TTG GTC TCC ATC T 3'

The primers shown were synthesized by MWG Biotech and the PCR reaction was carried out by the standard PCR method of Innis et al. (PCR Protocols. A Guide to Methods and Applications, 1990, Academic Press). The primers make it possible to amplify an approx. 3.8 kb DNA fragment carrying the pyc gene. The primers also contain the sequence for a cleavage site of the restriction endonuclease XbaI, which is indicated by brackets in the nucleotide sequence shown above.

The amplified DNA fragment of approx. 3.8 kb, carrying the pyc gene, was identified by gel electrophoresis in 0.8% agarose gel, isolated from the gel and purified by the conventional methods (QIAquick Gel Extraction Kit, Qiagen, Hilden).

The fragment was then ligated to vector pCRII-TOPO by means of the Dual Promotor Topo TA Cloning Kit (Invitrogen, Leek, The Netherlands, cat. number K4600-01). The ligation mixture was transformed to the E. coli strain TOP10 (Invitrogen, Leek, The Netherlands). Plasmid-carrying cells were selected by plating the transformation mixture on LB agar containing kanamycin (50 mg/l) and X-Gal (64 mg/l).

After isolation of the DNA, the plasmid obtained was checked by means of restriction cleavage and identified in agarose gel. The plasmid was called pCRII-TOPOpyc and the DNA sequence of the cloned insert was sequenced for control purposes. As the determined sequence of the pyc insert in pCRII-TOPOpyc matches the sequence of the gene library entry, this plasmid was used subsequently.

Example 4

Cloning of the dapB gene into vector pEC7

An approx. 1.1 kb DNA fragment carrying the dapB gene was isolated from plasmid pCR2.1TOPOdapB (from Example 2). For this purpose, plasmid pCR2.1TOPOdapB was fully digested with the restriction enzyme HindIII and the approx. 1.1 kb DNA fragment carrying the dapB gene was isolated.

The dapB fragment was inserted into vector pEC7. Vector pEC7 is based on E. coli - C. glutamicum shuttle vector pEC5 (Eikmanns et al., 102: 93-98 (1991)). The BamHI cleavage site not located in the polylinker was removed from plasmid pEC5 in the following manner: Plasmid pEC5 was partially cleaved with the restriction enzyme BamHI. The approx. 7.2 kb DNA fragment was isolated from the agarose gel and the protruding ends were filled in with Klenow polymerase (Boehringer Mannheim). The resulting DNA fragment was ligated (T4 ligase, Boehringer Mannheim). The ligation mixture was transformed to the E. coli strain

DH5α and kanamycin-resistant colonies were isolated on LB agar containing kanamycin (50 mg/l). Plasmid DNA was isolated from a transformant (QIAprep Spin Miniprep Kit from Qiagen) and checked by restriction cleavage with the restriction enzymes BamHI and PstI. The resulting plasmid was called pEC6.

Plasmid pEC6 was fully cleaved with the restriction enzyme XhoI. A DNA fragment carrying the trp terminator was ligated to vector DNA fragment (T4 ligase, Boehringer Mannheim). The ligation mixture was transformed to the E. coli strain DH5α and kanamycin-resistant colonies were isolated on LB agar containing kanamycin (50 mg/l). Plasmid DNA was isolated from a transformant (QIAprep Spin Miniprep Kit from Qiagen) and checked by restriction cleavage with the restriction enzymes BamHI and XhoI. The resulting plasmid was called pEC7.

The dapB-carrying DNA fragment obtained was ligated to vector pEC7 (T4 DNA ligase, Boehringer Mannheim), which had also been fully digested with the restriction enzyme HindIII and treated with alkaline phosphatase (Boehringer Mannheim). The ligation mixture was transformed to the E. coli strain DH5α and kanamycin-resistant colonies were isolated on LB agar containing kanamycin (50 mg/l). Plasmid DNA was isolated from a transformant (QIAprep Spin Miniprep Kit from Qiagen) and checked by restriction cleavage with the restriction enzyme HindIII. The resulting plasmid was called pEC7dapB (Figure 1). The Escherichia coli strain obtained was called DH5α/pEC7dapB.

Example 5

Cloning of the lysE gene into vector pEC7

Plasmid pUC18lysEneu described in Example 1 was fully digested with the restriction enzyme BamHI and the 1.1 kb BamHI fragment carrying the lysE gene was isolated as in Example 1. Vector pEC7 was likewise fully cleaved with

the restriction enzyme BamHI and treated with alkaline phosphatase. The BamHI vector fragment and the BamHI lysE fragment were ligated (Rapid DNA Ligation Kit, Boehringer Mannheim) and transformed to the E. coli strain DH5α. Plasmid-carrying transformants were selected on LB agar containing chloramphenicol (10 mg/l). Plasmid DNA was isolated (QIAprep Spin Miniprep Kit, Qiagen) and checked by restriction cleavage with the enzyme BamHI. The resulting plasmid was called pEC7lysE (Figure 2). The strain obtained by transformation of plasmid pEC7lysE to the E. coli strain DH5α was called DH5α/pEC7lysE.

Example 6

Cloning of the pyc gene into vector pEC7

The 3.8 kb DNA fragment carrying the pyc gene from C. glutamicum ATCC13032 was obtained from plasmid pCRII-TOPOpyc (from Example 3) by cleavage with the restriction enzyme XbaI. The 3.8 kb DNA fragment was identified by gel electrophoresis, isolated from the gel and purified by the conventional methods and the protruding ends were filled in with Klenow polymerase. Vector pEC7 was likewise fully cleaved with the restriction enzyme SmaI and treated with alkaline phosphatase. The SmaI vector fragment and the XbaI pyc fragment treated with Klenow polymerase were ligated (T4 ligase, Boehringer Mannheim) and transformed to the E. coli strain DH5α. Plasmid-carrying transformants were selected on LB agar containing chloramphenicol (10 mg/l). Plasmid DNA was isolated (QIAprep Spin Miniprep Kit, Qiagen, Hilden, Germany) and checked by restriction cleavage with the restriction enzyme SalI. The resulting plasmid was called pEC7pyc (Figure 3). The E. coli strain obtained by transformation of plasmid pEC7pyc to the E. coli strain DH5α was called DH5α/pEC7pyc.

Example 7

Preparation of a plasmid containing lysE and dapB genes

The dapB gene was isolated as a HindIII fragment from plasmid pCR2.1TOPOdapB containing the dapB gene from C. glutamicum ATCC13032. To do this, the plasmid was fully digested with the restriction enzyme HindIII and the dapB-carrying DNA fragment was isolated from 0.8% agarose gel (QIAquick Gel Extraction Kit, Qiagen).

Vector pEC7lysE was also fully digested with the restriction enzyme HindIII and treated with alkaline phosphatase. The 1.1 kb fragment containing dapB was ligated to the resulting linear vector fragment (T4 ligase, Boehringer Mannheim) and the ligation mixture was transformed to the E. coli strain DH5α. Plasmid-carrying transformants were selected on LB agar containing chloramphenicol (10 mg/l). Plasmid DNA was isolated (QIAprep Spin Miniprep Kit, Qiagen, Hilden, Germany) and checked by restriction cleavage with the restriction enzyme HindIII.

The resulting plasmid was called pEC7lysEdapB. This plasmid is capable of autonomous replication in Escherichia coli and in Corynebacterium and confers resistance to the antibiotic chloramphenicol on its host.

Plasmid pEC7lysEdapB simultaneously contains the dapB gene, which codes for dihydrodipicolinate reductase, and the lysE gene, which codes for the lysine exporter.

The strain obtained by the transformation of E. coli DH5α with pEC7lysEdapB was called DH5α/pEC7lysEdapB.

Example 8

Preparation of a plasmid simultaneously containing dapB and pyc genes

The plasmid carrying the pyc gene which codes for the pyruvate carboxylase from Corynebacterium glutamicum ATCC13032 was fully cleaved with the restriction enzyme XbaI and the protruding ends were filled in with Klenow polymerase as described in Example 6, making it possible to isolate the 3.8 kb DNA fragment containing the gene for pyruvate carboxylase.

Plasmid pEC7dapB (from Example 4) was also fully cleaved with the restriction enzyme SmaI and the ends were treated with alkaline phosphatase. The resulting linear vector fragment was ligated to the 3.8 kb DNA fragment containing the pyc gene using T4 DNA ligase (Boehringer Mannheim, Mannheim, Germany). This produced a plasmid containing both the dapB gene and the pyc gene from corynebacteria. Plasmid-carrying transformants were selected on LB agar containing chloramphenicol (10 mg/l). Plasmid DNA was isolated (QIAprep Spin Miniprep Kit, Qiagen, Hilden, Germany) and verified by restriction cleavage with the restriction enzyme SalI. The plasmid is shown in Figure 4 and was called pEC7dapBpyc. The E. coli strain obtained by transformation of plasmid pEC7dapBpyc to the E. coli strain DH5α was called DH5α/pEC7dapBpyc.

Example 9

Preparation of a plasmid containing sequences simultaneously coding for the lysE, dapB and pyc genes

Plasmid pCRII-TOPOpyc (from Example 3), which carries the pyc gene coding for the pyruvate carboxylase from Corynebacterium glutamicum ATCC13032, was fully cleaved with the restriction enzyme XbaI and treated with Klenow polymerase as described in Example 6, making it possible

to isolate the 3.8 kb DNA fragment containing the gene for pyruvate carboxylase.

Plasmid pEC7dapBlysE was also fully cleaved with the restriction enzyme SmaI and the ends were treated with alkaline phosphatase. The resulting linear vector fragment was ligated to the 3.8 kb DNA fragment containing the pyc gene using T4 DNA ligase (Boehringer Mannheim). This produces a plasmid containing the lysE gene and dapB gene and the pyc gene from Corynebacterium glutamicum. Plasmid-carrying transformants were selected on LB agar containing chloramphenicol (10 mg/l). Plasmid DNA was isolated (QIAprep Spin Miniprep Kit, Qiagen, Hilden, Germany) and verified by restriction cleavage with the restriction enzyme SmaI. The plasmid is shown in Figure 5 and was called pEC7lysEdapBpyc. The E. coli strain obtained by transformation of plasmid pEC7dapBlysEpyc to the E. coli strain DH5α was called DH5α/pEC7dapBlysEpyc.

Example 10

Transformation of the strain MH20-22B with plasmids pJC23 and pJC33

Plasmid pJC1 is a plasmid capable of replication in Escherichia coli and Corynebacterium glutamicum (Cremer et al., Molecular and General Genetics 220: 478-480 (1990)). Plasmid pJC33 (Cremer et al., Applied and Environmental Microbiology 57(6), 1746-1752 (1991)), which carries the lysC(Fbr) gene from the C. glutamicum strain MH20-22B, is derived therefrom.

Plasmid pJC23 is also based on vector pJC1 and carries the dapA gene from C. glutamicum ATCC13032 (Cremer et al., Molecular and General Genetics 220: 478-480 (1990)) (EP-B 0 435 132). Plasmids pJC1, pJC33 and pJC23 were introduced into the strain MH20-22B by the electroporation method (Haynes and Britz, FEMS Microbiology Letters (61) 329-334 (1989)). The C. glutamicum strain MH20-22B is an

AEC-resistant lysine producer deposited under the number DSM5715.

The transformants obtained by means of electroporation were isolated on selection agar (LBHIS agar (18.5 g/l of brain-heart infusion broth, 0.5 M sorbitol, 5 g/l of bacto tryptone, 2.5 g/l of bacto yeast extract, 5 g/l of NaCl, 18 g/l of bacto agar)) containing 15 mg/l of kanamycin. Plasmid DNA was isolated by the conventional methods (Peters-Wendisch et al., Microbiology 144, 915-927 (1998)), cleaved with suitable restriction endonucleases and checked. The strains obtained were called MH20-22B/pJC1, MH20-22B/pJC33 and MH20-22B/pJC23.

Example 11

Transformation with plasmids pEC7pyc, pEC7dapBpyc and pEC7lysEdapBpyc

The strains prepared in Example 10 were subsequently provided with a second plasmid.

The following plasmids were introduced by the electroporation method into the strains MH20-22B/pJC1, MH20-22B/pJC33 and MH20-22B/pJC23 described:

- pEC7pyc (cf. Example 6)
- pEC7dapBpyc (cf. Example 8)
- pEC7lysEdapBpyc (cf. Example 9)

The transformed bacteria are selected on the basis of the antibiotic resistance of the plasmids they contain. The transformants obtained by means of electroporation were isolated on selection agar (LBHIS agar containing 15 mg/l of kanamycin and 7.5 mg/l of chloramphenicol). Plasmid DNA was isolated, cleaved with suitable restriction endonucleases and checked.

The strains obtained are listed below:

DSM5715/pJC1/pEC7pyc
DSM5715/pJC33/pEC7pyc
DSM5715/pJC23/pEC7pyc
DSM5715/pJC23/pEC7dapBpyc
DSM5715/pJC23/pEC7lysEdapBpyc

Example 12

Preparation of L-lysine

The various C. glutamicum strains obtained in Examples 10 and 11 were cultivated in a nutrient medium suitable for lysine production and the lysine content of the culture supernatant was determined.

This was done by first incubating the various strains on agar plates with the appropriate antibiotics (brain-heart agar containing kanamycin (25 mg/l) and chloramphenicol (10 mg/l)) for 24 hours at 33°C. These agar plate cultures were used to inoculate a preculture (10 ml of medium in a 100 ml conical flask). Complete medium CgIII was used as the preculture medium. Kanamycin (25 mg/l) and chloramphenicol (10 mg/l) were added. The preculture was incubated for 24 hours at 33°C on a shaker at 250 rpm. This preculture was used to inoculate a main culture to give an initial OD (660 nm) of 0.2. Medium MM was used for the main culture.

Medium MM

CSL	5 g/l
MOPS	20 g/l
Glucose	50 g/l (autoclave separately)
Salts:	
$(NH_4)_2SO_4$	25 g/l
KH_2PO_4	0.1 g/l
$MgSO_4*7H_2O$	1.0 g/l
$CaCl_2*2H_2O$	10 mg/l
$FeSO_4*7H_2O$	10 mg/l
$MnSO_4*H_2O$	5.0 mg/l
Biotin	0.3 mg/l (sterile-filtered)
Thiamine*HCl	0.2 mg/l (sterile-filtered)
$CaCO_3$	25 g/l

Abbreviations:

CSL: corn steep liquor
MOPS: morpholinopropanesulfonic acid

CSL, MOPS and the salt solution are adjusted to pH 7 with aqueous ammonia and autoclaved. The sterile substrate and vitamin solutions and the dry-autoclaved $CaCO_3$ are then added.

Cultivation is carried out in a volume of 10 ml in a 100 ml conical flask with baffles. Kanamycin (25 mg/l) and chloramphenicol (10 mg/l) were added. Cultivation proceeded at 33°C and 80% atmospheric humidity.

After 72 hours the OD was measured at a wavelength of 660 nm. The amount of lysine formed was determined with an amino acid analyzer from Eppendorf BioTronik (Hamburg, Germany) by means of ion exchange chromatography and postcolumn derivation with ninhydrin detection. The glucose content was determined with a sugar analyzer from Skalar Analytik GmbH (Erkelenz, Germany).

The experimental results are shown in Table 1.

Table 1

Strain	Gene	OD (660 nm)	Lysine-HCl g/l
DSM5715/pJC1/pEC7pyc	pyc	9.3	11.3
DSM5715/pJC33/pEC7pyc	pyc, lysC(Fbr)	9.2	11.9
DSM5715/pJC23/pEC7pyc	pyc, dapA	9.3	14.4
DSM5715/pJC23/pEC7dapBpyc	pyc, dapA, dapB	9.3	16.9
DSM5715/pJC23/ pEC7lysEdapBpyc	pyc, dapA, dapB, lysE	9.1	17.6

Example 13

Cloning of the aecD gene into vector pUC18

Plasmid pSIR21 (Rossol, Thesis, University of Bielefeld 1992) was fully cleaved with the enzymes BglII and EcoRV and the 1.4 kb DNA fragment containing the aecD gene (accession number M89931) (Rossol and Pühler, Journal of Bacteriology 174 (9), 2968-2977 (1992)) from C. glutamicum ATCC13032 was isolated. The isolated DNA fragment was ligated to plasmid pUC18 (which had been fully digested with the enzymes BamHI and SmaI) using T4 DNA ligase, as described in Sambrook et al. (Molecular Cloning: A Laboratory Manual (1989), Cold Spring Harbor Laboratory Press). The ligation mixture was transformed to the E. coli strain DH5α. The transformants were selected on brain-heart agar plates containing 100 mg/l of ampicillin. Plasmid DNA was isolated from one colony. The plasmid obtained was called pUC18::aecD.

Example 14

Cloning of the dapA gene into plasmid pSP72

A dapA gene fragment is isolated from plasmid pJC20 (Cremer, J., Thesis 1989, University of Düsseldorf) as an SphI-BamHI fragment. Vector pSP72 (Promega Corporation, USA) was fully cleaved with the enzymes SphI and BamHI and treated with alkaline phosphatase. The dapA-carrying fragment was ligated to this vector using T4 DNA ligase. The DNA was then transformed to the E. coli strain XL1 Blue (Bullock, Fernandez and Short, BioTechniques (5), 376-379 (1987)). The transformants were selected on LB medium containing 100 mg/l of ampicillin. Plasmid DNA was isolated from one transformant and called pSP72::dapA.

Example 15

Mutagenesis of the dapA promoter and preparation of plasmids pSP72::dapA(MC20) and pSP72::dapA(MA16)

The Quickchange site directed mutagenesis kit from Stratagene was used for the mutagenesis of the promoter region. The following primers were constructed with the aid of said dapA sequence and used for the mutagenesis:

For the preparation of pSP72::dapA(MC20)

Primer dap1 for MC20
CCA AAT GAG AGA TGG TAA CCT TGA ACT CTA TGA GCA

Primer dap2 for MC20
GTG CTC ATA GAG TTC AAG GTT ACC ATC TTC CCT CAT TTG G

For the preparation of pSP72::dapA(MA16)

Primer dap3 for MA16
CCA AAT GAG GGA AGA AGG TAT AAT TGA ACT CTA TGA GCA

Primer dap4 for MA16
GTG CTC ATA GAG TTC AAT TAT ACC TTC TTC CCT CAT TTG G

The PCR was carried out as indicated by the manufacturer of the Quickchange site directed mutagenesis kit (Stratagene) using plasmid pSP72::dapA (from Example 14) as the template.

The mutagenesis mixtures were transformed to the E. coli strain XL1 Blue. The transformants were selected on LB medium containing 100 mg/l of carbenicillin. Plasmid DNA was isolated from one transformant and the loss of the BstEII cleavage site was controlled by BstEII digestion. Plasmids no longer carrying a BstEII cleavage site exhibited the desired mutation.

The plasmids obtained were transformed to the dapA-defective E. coli mutant RDA8. The transformation mixtures were plated on LB containing 100 mg/l of carbenicillin in order to test the complementation of the dapA mutation. DNA was isolated from one transformant in each case and the plasmids obtained were called pSP72::dapA(MC20) and pSP72::dapA(MA16). The plasmids were sequenced by the chain termination method described in Sanger et al., Proceedings of the National Academy of Sciences of the USA (74), 5463-5467 (1977), using the reverse and universal sequencing primers. The sequencing reaction was performed with the aid of the AutoRead Sequencing Kit (Pharmacia, Freiburg). The electrophoretic analysis and detection of the sequencing products were carried out with the A.L.F. DNA sequencer (Pharmacia, Freiburg, Germany).

Example 16

Preparation of plasmids pK19mobsacBaecD::dapA(MC20) and pK19mobsacBaecD::dapA(MA16) (recloning of the mutagenized fragments)

Plasmids pSP72::dapA(MC20) and pSP72::dapA(MA16) (from Example 15) were fully cleaved with the restriction enzymes PvuII and SmaI. The 1450 bp PvuII-SmaI fragments

carrying the dapA gene with the mutated MC20 or MA16 promoter were ligated to StuI-cleaved vector pUC18::aecD (from Example 13) using T4 DNA ligase. The ligation mixture was transformed to the E. coli strain DH5α. The transformants were selected on LB medium containing 100 mg/l of ampicillin. Plasmid DNA was isolated from one transformant in each case to give plasmids pUC18aecD::dapA(MC20) and pUC18aecD::dapA(MA16).

Plasmids pUC18aecD::dapA(MC20) and pUC18aecD::dapA(MA16) were partially cleaved with the restriction enzyme EcoRI and fully cleaved with the enzyme SalI to give the 3.0 kb fragment carrying aecD::dapA(MA16) or aecD::dapA(MC20). The fragment was ligated to vector pK19mobsacB (which had been cleaved and treated with alkaline phosphatase) (Schäfer et al., Gene (145), 69-73 (1994)) using T4 DNA ligase. The ligation mixture was transformed to the E. coli strain DH5 (Hanahan (1985), in: DNA Cloning. A Practical Approach, Vol. I, IRL-Press, Oxford, Washington DC, USA). The transformants were selected on LB medium containing 50 mg/l of kanamycin. Plasmid DNA was isolated from one transformant in each case to give plasmids pK19mobsacBaecD::dapA(MC20) and pK19mobsacBaecD::dapA(MA16).

The plasmid DNA was transformed to the E. coli strain S17-1 (Simon, Priefer and Pühler, Bio/Technology (1), 784-791 (1983)). The transformants were selected on LB medium containing 50 mg/l of kanamycin. Plasmid DNA was isolated from one transformant in each case and checked. The strains obtained were called S17-1/pK19mobsacBaecD::dapA(MC20) and S17-1/pK19mobsacBaecD::dapA(MA16).

Example 17

Preparation of the C. glutamicum strains DSM5715aecD::dapA(MC20) and DSM5715aecD::dapA(MA16)

Plasmids pK19mobsacBaecD::dapA(MC20) and pK19mobsacBaecD::dapA(MA16) were transferred from S17-1/pK19mobsacBaecD::dapA(MC20) and S17-1/pK19mobsacBaecD::dapA(MA16) (from Example 16) to the C. glutamicum strain DSM5715 by the conjugation method (Schäfer et al., Journal of Bacteriology (172), 1663-1666 (1990)). For selection of the transconjugants, the conjugation mixtures were plated on brain-heart medium containing nalidixic acid and kanamycin. The transconjugants obtained were incubated overnight in 10 ml of brain-heart medium. Aliquots were then plated on plates containing sucrose (brain-heart agar containing 10% of sucrose) in order to select for loss of sucrose sensitivity. Sucrose-resistant clones were isolated and checked again on agar plates containing chloramphenicol and kanamycin (brain-heart medium containing 15 mg/l of kanamycin and brain-heart medium containing 10 mg/l of chloramphenicol).

Colonies exhibiting the following phenotype were isolated:

sucrose resistant
kanamycin sensitive
chloramphenicol sensitive

The insertion of the dapA gene fragment into the aecD gene was checked by the Southern blot method (Sambrook et al., Molecular Cloning: A Laboratory Manual (1989), Cold Spring Harbor Laboratory Press).

Example 18

Preparation of the C. glutamicum strains DSM5715aecD::dapA(MC20)/pEC7pyc, DSM5715aecD::dapA(MA16)/pEC7pyc, DSM5715aecD::dapA(MC20)/pEC7, DSM5715aecD::dapA(MA16)/pEC7 and DSM5715/pEC7

As described in Example 6, the pyc gene is present in vector pEC7pyc. This plasmid pEC7pyc and plasmid pEC7 were introduced into the strains DSM5715aecD::dapA(MC20), DSM5715aecD::dapA(MA16)and DSM5715 (from Example 17) by means of electroporation (Haynes 1989, FEMS Microbiology Letters 61, 329-334) to give C. glutamicum DSM5715aecD::dapA(MC20)/pEC7pyc, DSM5715aecD::dapA(MA16)/pEC7pyc, DSM5715aecD::dapA(MC20)/pEC7, DSM5715aecD::dapA(MA16)/pEC7 and DSM5715/pEC7. The transformants were selected on brain-heart agar containing 25 mg/l of kanamycin. Plasmid DNA was isolated from one transformant in each case and checked.

The following strains were obtained in this way:

DSM5715aecD::dapA(MC20)/pEC7pyc,
DSM5715aecD::dapA(MA16)/pEC7pyc,
DSM5715aecD::dapA(MC20)/pEC7,

DSM5715aecD::dapA(MA16)/pEC7 and

DSM5715/pEC7.

Example 19

Preparation of lysine with the strains prepared in Example 18

After precultivation in CgIII medium (Kase & Nakayama, Agricultural and Biological Chemistry 36 (9), 1611-1621 (1972)), the strains DSM5715aecD::dapA(MC20)/pEC7pyc,

DSM5715aecD::dapA(MA16)/pEC7pyc, DSM5715aecD::dapA(MC20)/pEC7, DSM5715aecD::dapA(MA16)/pEC7 and DSM5715/pEC7 were cultivated in MM production medium as described in Example 12. After incubation for 48 hours, the optical density at 660 nm and the concentration of L-lysine formed were determined.

The experimental results are shown in Table 2.

Table 2

Strain	OD (660 nm)	Lysine·HCl g/l
DSM5715aecD::dapA(MC20)/pEC7pyc	14.15	13.3
DSM5715aecD::dapA(MA16)/pEC7pyc	13.6	13.4
DSM5715/pEC7	13.3	11.5
DSM5715aecD::dapA(MA16)/pEC7	13.3	12.9
DSM5715aecD::dapA(MC20)/pEC7	14.0	12.8

Example 20

Transformation of strain DM678 with plasmids pEC7 and pEC7lysEdapBpyc

The lysine producing C. glutamicum strain DM678 was derived from ATCC13032 by several rounds of mutagenesis and screening. It has a partial requirement for L-threonine and its growth is sensitive towards L-methionine. Strain DM678 is deposited as DSM12866 at the Deutsche Sammlung für Mikroorganismen und Zellkulturen (DSMZ, Braunschweig, Deutschland).

Plasmids pEC7 (from Example 4) and pEC7lysEdapBpyc (from Example 9) were introduced into the strain DM678 by the electroporation method (Haynes and Britz, FEMS Microbiology Letters (61) 329-334 (1989)).

The transformants obtained by means of electroporation were isolated on selection agar (LBHIS agar (18.5 g/l of brain-heart infusion broth, 0.5 M sorbitol, 5 g/l of bacto tryptone, 2.5 g/l of bacto yeast extract, 5 g/l of NaCl, 18 g/l of bacto agar)) containing 7.5 mg/l of chloramphenicol. Plasmid DNA was isolated by the conventional methods (Peters-Wendisch et al., Microbiology 144, 915-927 (1998)), cleaved with suitable restriction endonucleases and checked by agarosegel electrophoresis. The strains obtained were called DM678/pEC7 and DM678/pEC7lysEdapBpyc.

Example 21

Preparation of L-lysine

The C. glutamicum strains obtained in Examples 20 were cultivated in a nutrient medium suitable for lysine production and the lysine content of the culture supernatant was determined.

This was done by first incubating the strains on agar plates (brain-heart) with chloramphenicol (10 mg/l) for 24 hours at 33°C. These agar plate cultures were used to inoculate a preculture (10 ml of medium in a 100 ml conical flask). Complete medium CgIII which had been supplemented with 10 mg/l of chloramphenicol was used as the preculture medium. The preculture was incubated for 24 hours at 33°C on a shaker at 250 rpm. This preculture was used to inoculate a main culture to give an initial OD (660 nm) of 0.1. Medium MM2 was used for the main culture.

Medium MM2:

CSL	25 g/l
MOPS	20 g/l
Glucose	80 g/l (autoclave separately)
Salts:	
$(NH_4)_2SO_4$	25 g/l
KH_2PO_4	0.1 g/l
$MgSO_4*7H_2O$	0.25 g/l
$CaCl_2*2H_2O$	10 mg/l
$FeSO_4*7H_2O$	10 mg/l
$MnSO_4*H_2O$	10 mg/l
Biotin	0.3 mg/l (sterile-filtered)
Thiamine*HCl	0.2 mg/l (sterile-filtered)
$CaCO_3$	25 g/l (autoclave separately)

Abbreviations:

CSL: corn steep liquor
MOPS: morpholinopropanesulfonic acid

CSL, MOPS and the salt solution are adjusted to pH 7 with aqueous ammonia and autoclaved. The sterile substrate and vitamin solutions, the Chloramphenicol (10 mg/l) and the dry-autoclaved $CaCO_3$ are then added.

Cultivation is carried out in a volume of 10 ml in a 100 ml conical flask with baffles. Incubation proceeded at 33°C and 80% atmospheric humidity.

After 48 hours the OD was measured at a wavelength of 660 nm. The amount of lysine formed was determined with an amino acid analyzer from Eppendorf BioTronik (Hamburg, Germany) by means of ion exchange chromatography and postcolumn derivation with ninhydrin detection. The glucose content was determined with a sugar analyzer from Skalar Analytik GmbH (Erkelenz, Germany).

The experimental results are shown in Table 3.

Table 3

Strain	Gene	OD (660 nm)	Lysine-HCl g/l
DM678/pEC7		25.6	16.7
DM678/ pEC7lysEdapBpyc	pyc, dapB, lysE	23.3	17.7

Brief Description of the Figures

Figure 1 illustrates the pEC7dapB plasmid (cf. Example 4)

Figure 2 illustrates the pEC7lysE plasmid (cf. Example 5)

Figure 3 illustrates the pEC7pyc plasmid (cf. Example 6)

Figure 4 illustrates the pEC7dapBpyc plasmid (cf. Example 8)

Figure 5 illustrates the pEC7lysEdapBpyc plasmid (cf. Example 9).

The abbreviations used in the Figures are defined as follows:

Cm: chloramphenicol resistance gene

dapB: dapB gene from C. glutamicum

lysE: lysE gene from C. glutamicum

pyc: pyc gene from C. glutamicum

OriE: plasmid-coded origin of replication of E. coli

pBL: DNA fragment of plasmid pBL1

EcoRI: cleavage site of the restriction enzyme EcoRI

EcoRV: cleavage site of the restriction enzyme EcoRV

HincII: cleavage site of the restriction enzyme HincII

HindIII: cleavage site of the restriction enzyme HindIII

KpnI: cleavage site of the restriction enzyme KpnI

SalI: cleavage site of the restriction enzyme SalI

SmaI: cleavage site of the restriction enzyme SmaI

SphI: cleavage site of the restriction enzyme SphI

PvuII: cleavage site of the restriction enzyme PvuII

BamHI: cleavage site of the restriction enzyme BamHI